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Phytochemical content and antioxidant activity of six diverse varieties of whole wheat

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ABSTRACT

The phytochemical content and antioxidant activity of six diverse varieties of whole wheat are reported. The free phenolic content ranged from 255 (KanQueen) to 499 (Roane) μmol gallic acid equivalents/100 g DW. The bound phenolic content ranged from 582 (Roane) to 662 (Cham1) μmol gallic acid equivalents/100 g DW. The bound fraction contributed 53.8–69.7% of the total phenolic content of the wheat varieties analysed. Ferulic acid was the predominant phenolic acid found in whole wheat. Total ferulic acid content ranged from 310.8 (Caledonia) to 496.1 (KanQueen) μmol ferulic acid/100 g DW. The percentage of ferulic acid found in the insoluble-bound fraction ranged from 87.4% (Caledonia) to 97.2% (KanQueen). Other phenolic acids, *p*-coumaric acid, syringic acid, vanillic acid, and caffeic acid were also detected. Lutein was the predominant carotenoid found in the whole wheat varieties analysed. Zeaxanthin, β-carotene, and β-cryptoxanthin were also detected. Mainly α- and β-tocopherols and α- and β-tocotrienols were found in all varieties of whole wheat though γ-tocopherol was detected in all but two varieties. β-Tocotrienol was the predominant form of vitamin E found in all varieties of whole wheat. The antioxidant activity was assessed using the oxygen radical absorbance capacity (ORAC) assay. The ORAC of the free fraction ranged from 1958 to 3749 μmol Trolox equivalents/100 g DW. The ORAC of the bound fraction ranged from 3190 to 5945 μmol Trolox equivalents/100 g DW. Total phenolic content was correlated with oxygen radical absorbance capacity ($R^2 = 0.810$; $p < 0.001$). The phytochemicals found in whole grains may be responsible for the health benefit of whole grain consumption.

1. Introduction

Whole grain consumption has been consistently associated with reduced risk of developing chronic diseases, including cardiovascular disease (Anderson, 2004; Jacobs, Meyer, Kushi, & Folsom, 1998), type II diabetes (Montonen, Knekt, Jarvinen, Aromaa, & Reunanen, 2003), obesity (Liu et al., 2003), and cancer (Jacobs, Slavin, & Marquart, 1995; Schatzkin et al., 2007). For this reason, the Dietary Guidelines for Americans recommend consumption of at least three-ounce equivalents of whole grains per day and to make sure at least half of total grain product consumption comes from whole grain products (USDA, 2005).

Phytochemicals are the bioactive, non-nutrient, naturally occurring plant compounds found in fruits, vegetables, and whole grains (Liu, 2004). The health benefit of whole grain consumption may be due to the unique phytochemicals found in whole grains (Liu, 2007). Whole grain phytochemicals include phenolics, compounds containing one or more aromatic rings and one or more hydroxyl groups, carotenoids, and vitamin E, amongst others. Previously, the phenolic content of whole grains had been underestimated, as most research only determined the free phenolic content and not the content of phenolics that were bound to cell wall materials (Adom & Liu, 2002). Bound phytochemicals cannot be digested by human enzymes, could survive stomach and small intestine digestion, and therefore may possibly reach the colon. The colonic microflora may release the bound phytochemicals through fermentation, and thus provide site-specific health benefits in colon or other tissues after absorption (Liu, 2007).

Whole grain phytochemicals have antioxidant activity, the ability to scavenge free radicals that may oxidise biologically relevant molecules (Liu, 2007). The antioxidant activity of whole grains has

Abbreviations: DW, dry weight; FW, fresh weight; HPLC, high performance liquid chromatography; ORAC, oxygen radical absorbance capacity.

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been determined using many different antioxidant activity assays (Adom & Liu, 2005; Adom, Sorrells, & Liu, 2003; Liyana-Pathirana & Shahidi, 2004, 2005; Moore et al., 2005; Mpofu, Sapirstein, & Beta, 2006; Pellegrini et al., 2006). The oxygen radical absorbance capacity (ORAC) assay measures a sample's ability to prevent the oxidation of fluoroscein by a peroxyl radical induced by ABAP compared to the ability of various concentrations of Trolox (Ou, Hampsch-Woodill, & Prior, 2001) and has been proposed as a standard antioxidant activity assay to measure the antioxidant activity of foods (Prior, Wu, & Schaich, 2005).

Wheat is an important crop for the US economy. In 2007–2008, more than 60 million acres of wheat were planted yielding a projected 2 billion bushels of wheat in the US (Vocke & Allen, 2007). Several different food products are made from different varieties of wheat. Hard wheat is generally used in the production of breads and cakes. Soft wheat is generally used in the production of cookies, crackers, and breakfast foods. Durum wheat, which tends to have a harder kernel than other types of wheat, is usually used in the production of pastas. The differences in hardness of the kernel, and subsequently the type of products produced from them, is due to their gluten content (Distaam & Carcea, 2001).

Previous studies have reported the phytochemical content of whole wheat. Adom et al. (2003) reported the phytochemical content and antioxidant activity of 11 diverse varieties of wheat. However, the content and distribution of vitamin E, a group of lipophilic antioxidants associated with health benefit and common in grains, was not determined. Further, the antioxidant activity of the whole wheat samples was determined using the total oxyradical scavenging capacity (TOSC) assay. Moore et al. (2005) reported the carotenoid, tocopherol, phenolic acid, and antioxidant activity of eight Maryland-grown soft wheat samples. However, the contributions from the free and bound fractions to the total phenolic content and antioxidant activity and tocotrienols were not reported. Further, only soft wheat samples were used in this study. The phytochemical content and antioxidant activity of other varieties of wheat were not determined. Mpofu et al. (2006) reported the phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. However, no distinction was made between the free and bound fractions of whole wheat. Pellegrini et al. (2006) reported the antioxidant activity of whole wheat. Though the authors did make note the use of durum wheat, no mention was made of the variety of wheat used in the study. Further, the ferric ion reducing antioxidant power (FRAP) assay, total peroxyl radical-trapping antioxidant parameter (TRAP) assay, and trolox equivalent antioxidant capacity (TEAC) assays were used to determine the free and bound antioxidant activity of the wheat samples (Benzie & Szeto, 1999; Ghiselli, Serafini, Maiani, Azzini, & Ferroluzzi, 1995; Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993). Liyana-Pathirana and Shahidi (2006) reported the total phenolic content and antioxidant activity of hard and soft wheat. Though the authors did use the ORAC assay to distinguish between the free and bound antioxidant activity of whole wheat, a diverse range of wheat varieties was not used in the study.

The content of whole grain phytochemicals needs closer examination due to their potential health benefit in the prevention of chronic diseases. A more complete analysis of the phytochemical content and antioxidant activity of a range of diverse whole wheat samples is needed. Emphasis on the distribution of free and bound phenolic content and antioxidant activity is key to understanding the potential health benefit of whole grain consumption. The distinction between free and bound antioxidant activity needs to be made in order to understand the potential benefit of whole grain consumption. The objective of this study was to determine the phytochemical profiles and antioxidant activity of six diverse varieties of wheat.

2. Materials and methods

2.1. Chemicals and reagents

Sodium hydroxide, sodium sulphate, and hexanes were purchased from Fisher Scientific (Pittsburgh, PA). Acetone, sodium chloride, sodium carbonate, ethanol, ethyl acetate, and trifluroacetic acid were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Folin–Ciocalteu reagent, ferulic acid, *p*-coumaric acid, caffeic acid, *p*-hydroxybenzoic acid, carotene (α:β, 1:2), vanilic acid, syringic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), pyrogallol, and lutein were purchased from Sigma (St. Louis, MO). Gallic acid was purchased from ICN Biomedicals, Inc. (Aurora, OH). The compound 2,2′-azobis(2-amidinopropane)dihydrochloride (ABAP) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Zeaxanthin and β-cryptoxanthin were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ). All tocopherols and tocotrienols were purchased from Calbiochem (Darmstadt, Germany).

2.2. Grain samples and sample preparation

Wheat varieties (Table 1) were provided by Dr. Mark E. Sorrells of the Cornell Small Grains Breeding and Genetics Program in the Department of Plant Breeding and Genetics at Cornell University (Ithaca, NY). All wheat varieties were milled to a fine powder using a 20, 40, and 60 mesh size screen successively and mixed thoroughly. All samples were stored at −20 °C until analysis within one month. The moisture content of all samples was determined by taking the weight of the samples before and after drying at 105 °C for 16 h and used to express phytochemical contents to a dry weight basis (AOAC, 1990).

2.3. Extraction of free phenolics

Free phenolics of wheat samples were extracted using a modification of the method previously reported by Adom and Liu (2002) and Adom et al. (2003). Briefly, 1 g of whole-wheat flour was blended with 50 mL of 80% chilled acetone. The mixture was then centrifuged at 2500*g* for 10 min. The supernatant was removed and the remaining pellet was again extracted with 50 mL of 80% chilled acetone. The supernatants were pooled and evaporated at 45 °C to dryness. The solution was then reconstituted in methanol/hydrochloric acid (1 M; 85:15, v/v), filtered through a 0.45 μm filter, and stored at −40 °C until analysis within three days.

2.4. Extraction of bound phenolics

Bound phenolics of wheat samples were extracted using a modification of the method previously reported by our laboratory (Adom and Liu, 2002; Adom et al., 2003). Briefly, insoluble-bound phenolics were extracted from the residue from the free phenolic extraction. The residue was first digested with 2 M sodium hydroxide at room temperature for 1 h whilst shaking under nitrogen. The mixture was then neutralised with concentrated hydrochloric acid.

Table 1
Descriptions of the six wheat varieties used in this study.

Variety	Description	References
Stoa	Hard Red Spring	North Dakota AES (1984)
Roane	Soft Red Winter	Griffey et al. (2001)
Caledonia	Soft White Winter	Sorrells and Cox (2003)
Foster	Soft Red Winter	VanSanford et al. (1997)
Cham1	White Spring Durum	Nachit et al. (2001)
KanQueen	Semi-Hard Red Winter	Bayles and Clark (1954)

Hexanes were used to extract lipids in the mixture. The remaining mixture was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness. The phenolics were reconstituted in methanol/hydrochloric acid (1 M; 85:15, v/v), filtered through a 0.45 μm filter, and stored at −40 °C until analysis within three days.

2.5. Extraction of soluble-conjugated phenolic compounds

Soluble-conjugated phenolic compounds were extracted from free phenolic extracts using the method routinely used in our laboratory (Adom and Liu, 2002). Free phenolics extracts were digested with 2 M sodium hydroxide for 1 h at room temperature under nitrogen gas, and then neutralised with concentrated hydrochloric acid. The mixture was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness under nitrogen at 35 °C. The remaining residue was dissolved in methanol/hydrochloric acid (1 M; 85:15, v/v), filtered through a 0.45 μm filter, and stored at −40 °C until analysis within three days.

2.6. Determination of total phenolic content

Total phenolic content of each wheat sample was determined using the colorimetric method described by Singleton, Orthofer, and Lamuela-Raventos (1999) and modified in our laboratory (Adom, Sorrells, & Liu, 2005; Dewanto, Wu, & Liu, 2002). Briefly, extracts were reacted with Folin–Ciocalteu reagent and then neutralised with sodium carbonate. After 90 min, the absorbance of the resulting solution was measured at 760 nm. Gallic acid was used as the standard and total phenolic content was expressed as μmol of gallic acid equivalents/100 g sample DW.

2.7. Determination of phenolic acid composition

The determination of the phenolic composition was done using an r*p*-HPLC-DAD method. Briefly, the mobile phase [water to pH 2.8 with acetic acid (A) and acetonitrile/water 70:30 v/v to pH 2.8 with acetic acid (B)] was delivered using a Waters 600E quaternary pump at a flow rate of 1.5 mL/min using the following gradient program: linear gradient from 0/100% to 10/90% B/A for 2.5 min, linear gradient from 10/90% to 12/88% B/A for 3.5 min, linear gradient from 12/88% to 23/77% B/A for 10 min, linear gradient from 23/77% to 95/5% B/A for 4 min, and linear gradient from 95/5% to 0/100% B/A for 6 min. The total run time was 20 min with a 6 min delay between injections. Seventy-five microlitres of sample were injected using a Water 717 autosampler. Separation of phenolic compounds was done using a C18 column (5 μm, 250 mm × 4.6 mm; Grace Vydac, Baltimore, MD). Phenolic compounds were detected using a Waters 996 Photodiode Array Detector. Each injection was monitored at 282 nm. Identification of each peak was confirmed using the retention time and absorbance spectrum of each pure compound. Percent recoveries were determined by spiking a known amount of pure compound into a sample and performing the same extraction and analytical procedures. The percent recovery for ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, caffeic acid, and *p*-hydroxybenzoic acid were 101.6 ± 9.9, 99.3 ± 7.8, 83.0 ± 7.3, 85.0 ± 3.7, 89.3 ± 8.1, and 93.0 ± 7.4, respectively. The detection limits of all phenolic acids were less than 0.3 μg per injection. Data signals were acquired and processed using Waters Empower software (2002) (Waters Corp., Milford, MA).

2.8. Extraction of carotenoids

Carotenoids were extracted using the method described by Hentschel et al. (2002) and modified in our laboratory (Liu, Glahn, & Liu, 2004). The extraction was performed under dim lighting and all sample tubes were wrapped in lightproof paper to protect carotenoids from light-induced degradation. Samples and 60 mg magnesium carbonate were placed in a 10 × 120 mm screw-cap test tube to which 3 mL methanol/tetrahydrofuran (1:1, v/v) were added. The sample was vortexed, placed in a 75 °C water bath for 5 min, vortexed again and immediately centrifuged at 2000*g* for 5 min. After the upper solvent layer was removed to a second test tube, three additional washes of 3 mL methanol/tetrahydrofuran (1:1, v/v) were performed for complete extraction of carotenoids. Upper solvent extraction layers were pooled and vortexed with 1.5 g sodium sulphate. The solvent layer was removed and the residual rinsed at least twice with 2 mL hexane. The combined hexane solvent was evaporated to dryness under a gentle stream of nitrogen. The dry residue was re-dissolved with 0.5 mL methanol/tetrahydrofuran (1:1, v/v) and stored under nitrogen at −20 °C until HPLC analysis within two days.

2.9. Determination of carotenoid content

Carotenoid content was determined using the method described by Hentschel et al. (2002) modified by our laboratory (Liu et al., 2004). Briefly, the carotenoid content of each sample was determined using an r*p*-HPLC procedure employing a 250 × 4.6 mm YMC C30 column, 3 μm particle size (YMC, Waters Inc., Wilmington, NC). The mobile phase [methanol/water (95:5, v/v, A) and methyl tert-butyl ether (B)] was delivered at 1.9 mL/min using a Water 515 HPLC pump (Water Corp., Milford, MA). The gradient program was as follows: 25/75% B/A for 11 min, 25/75%–30/70% B/A for 2 min, 30/70% B/A for 9 min, 30/70%–25/75% B/A for 1 min and then 25/75% B/A for at least 5 min where the next injection would occur. A Waters 2487 dual wavelength absorbance detector (Waters Corps, Milford, MA) was used for UV detection of analytes at 450 nm. Data signals were acquired and processed on a PC running the Waters Millennium software, version 3.2 (1999) (Waters Corp, Milford, MA). Percent recoveries for all carotenoids were greater than 94% (Liu et al., 2004). The carotenoid content of each sample extract was extrapolated from a pure carotenoid standard curve. All samples were injected via a 20 μL loop and peak heights were used for all calculations. Data were expressed as μg/100 g DW.

2.10. Extraction of vitamin E

Extraction of tocopherols and tocotrienols from whole wheat was done using a modification of the method described by Panfili, Fratianni, and Irano (2003). Briefly, 2 mL 95% ethanol, 1 mL 300 mM sodium chloride, 4 mL 500 mM pyrogallol in ethanol, 1 mL 1 M ascorbic acid, then 2 mL 10.7 M potassium hydroxide were added to 1 g sample in a glass screw-cap tube. The headspace was flushed with nitrogen and the sample was digested for 45 min at 75 °C in a constantly agitated water bath. After 45 min, the tubes were cooled to room temperature in an ice bath. After addition of 750 μL 3 M sodium chloride and 8 mL hexane/ethyl acetate (9:1, v/v), the solution was then sonicated for 10 min, and the vitamin E extracted for 5 min. The tube was then centrifuged at 100*g* for 1 min and the organic layer was removed. The extraction procedure was repeated twice and the organic layers from each extraction were pooled. The pooled organic layers were transferred to a separatory funnel and washed twice with 10 mL distiled water. After transfer to an Erlenmeyer flask, the solution was dehydrated with 5 g sodium sulphate. The solution was then filtered using glass wool and evaporated to dryness under a gentle stream of nitrogen at 35 °C. The residue was dissolved in 2 mL hexane/ethyl acetate/acetic acid (99.0:0.5:0.5,

v/v/v), capped under nitrogen and stored at −20 °C until HPLC analysis within seven days.

2.11. Determination of vitamin E content

Chromatographic separation of tocopherols and tocotrienols was done using a n*p*-HPLC with a fluorescence detector. Briefly, the mobile phase, hexanes/ethyl acetate/acetic acid (99.0:0.5:0.5, v/v/v) was filtered using a 0.45 μm filter and delivered by a Waters 501 Solvent Delivery System at a flow rate of 1 mL/min. One hundred microlitres of sample were injected into the system and a silica column (Grace Vydac 250 × 4.6 mm, 5 μm particle size; Grace Vydac Carlsbad, CA) was used to separate the vitamers. Detection of tocopherols and tocotrienols was conducted using fluorescence detection with a Waters 474 Scanning Fluorescent Detector at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Data signals were acquired and processed on a PC running the Waters Millennium Software, version 3.2 (1999) (Waters Corp, Miford, MA). The detection limits for α-tocopherol and α-tocotrienol were 0.1 ng per injection. The percent recoveries for α-tocopherol and α-tocotrienol in spiked samples were 95.2 ± 5.3 and 99.9 ± 9.9, respectively. The detection limits and percent recoveries for all other vitamers were assumed to be on the same order. Total vitamin E content was determined to be the sum of all eight vitamers and expressed as μg/g DW.

2.12. Antioxidant activity

The antioxidant activity was determined using oxygen radical absorbance capacity (ORAC) assay described by Ou et al. (2001) and modified in our laboratory (Wolfe et al., 2008; Wolfe & Liu, 2007, 2008). Phenolic extract dilutions were prepared with 75 mM phosphate buffer (pH 7.4). The assay was performed in black-walled 96-well plates (Corning Scientific, Corning, NY). The outside wells of the plate were not used as there was much more variation from them than from the inner wells. Each well contained 20 μL extracts or 20 μL Trolox standard (range 6.25–50 μM), and 200 μL fluoroscein (final concentration 0.96 μM), which were incubated at 37 °C for 20 min. After incubation, 20 μL of 119 mM ABAP was added to each well. Fluorescence intensity was measured using Fluoroskan Ascent FL plate-reader (Thermo Labsystems, Franklin, MA) at excitation of 485 nm and emission of 520 nm for 35 cycles every 5 min. ORAC was expressed as μmol Trolox equivalents/100 g DW.

2.13. Statistical analysis

Data were reported as mean ± standard deviation for triplicate determinations of each sample. ANOVA and Tukey's comparison test were performed using Minitab Statistical Software version 15 (State College, PA) to identify differences between values. Statistical significance was defined to be at a level of $p < 0.05$.

3. Results

3.1. Total phenolic content

The free phenolic content ranged from 254.7 (KanQueen) to 499.5 (Roane) μmol gallic acid equivalents/100 g DW. The ratio of bound phenolics to free phenolic ranged from 1.2 (Roane) to 2.3 (KanQueen). The bound phenolic content ranged from 581.9 (Roane) to 662.4 (Cham1) μmol gallic acid equivalents/100 g DW. The total phenolic content ranged from 841 (KanQueen) to 1099 (Stoa) μmol gallic acid equivalents/100 g DW (Table 2). The total phenolic content of Stoa, Roane, and Cham1 were significantly different ($p < 0.05$) from the total phenolic content of Caledonia, Foster, and KanQueen.

3.2. Phenolic acid composition

Ferulic acid was the predominant phenolic acid found in each variety of whole wheat and was found in the free, soluble-conjugated, and insoluble-bound fractions (Table 3). Free ferulic acid content ranged from 4.3 (Foster) to 20.1 (Roane) μmol ferulic acid/100 g DW. Soluble-conjugated ferulic acid ranged from 9.6 (KanQueen) to 30.5 (Caledonia) μmol ferulic acid/100 g DW. The soluble-conjugated ferulic acid content of Caledonia was significantly different ($p < 0.05$) from all other soluble-conjugated ferulic acid contents. The bound ferulic acid content ranged from 271.5 (Caledonia) to 482.1 (KanQueen) μmol ferulic acid/100 g DW. The bound ferulic acid content of KanQueen was significantly different ($p < 0.05$) from all other bound ferulic acid contents. Total ferulic acid content ranged from 310.8 (Caledonia) to 496.1 (KanQueen) μmol ferulic acid/100 g DW. The percentage of ferulic acid found in the insoluble-bound fraction ranged from 87.4% (Caledonia) to 97.2% (KanQueen).

p-Coumaric acid was found in the free, soluble-conjugated, and insoluble-bound fractions. Free *p*-coumaric acid content ranged from 9.2 (Foster) to 25.4 (Caledonia) μmol *p*-coumaric acid/100 g DW. The free *p*-coumaric acid of Caledonia was significantly different ($p < 0.05$) from all other free *p*-coumaric acid contents. Soluble-conjugated *p*-coumaric acid ranged from 7.4 (Stoa) to 10.0 (Caledonia) μmol *p*-coumaric acid/100 g DW. Insoluble bound *p*-coumaric acid content ranged from 15.9 (Cham1) to 29.0 (KanQueen) μmol *p*-coumaric acid/100 g DW. The bound *p*-coumaric acid content of KanQueen was significantly different ($p < 0.05$) from all other bound *p*-coumaric acid contents. The percentage of *p*-coumaric acid found in the insoluble-bound fraction ranged 32.3% (Caledonia) to 63.4% (KanQueen).

Syringic acid was found in the soluble-conjugated and insoluble-bound fractions. Soluble-conjugated syringic acid content ranged from 4.9 (Cham1) to 11.5 (Caledonia) μmol syringic acid/100 g DW. Insoluble-bound syringic acid content ranged from 3.1 (Caledonia) to 9.8 (KanQueen) μmol syringic acid/100 g DW. The bound syringic acid content of KanQueen was significantly different ($p < 0.05$) from all other bound syringic acid contents. No insoluble-bound syringic acid was detected in Foster. The percentage of syringic acid in the insoluble-bound fraction ranged from 21.2% (Caledonia) to 63.2% (KanQueen).

Vanillic acid was found only in the soluble-conjugated fraction. Soluble-conjugated vanillic acid contents ranged from 4.3 (KanQueen) to 8.3 (Caledonia) μmol vanillic acid /100 g DW.

Caffeic acid was only found in the insoluble-bound fraction. Caffeic acid contents ranged from 3.2 (Caledonia) to 7.4 (KanQueen) μmol caffeic acid/100 g DW. The bound caffeic acid content of KanQueen was significantly different ($p < 0.05$) from all other bound caffeic acid contents.

Table 2
Total phenolic content of wheat varieties. Values expressed as μmol gallic acid equivalents/100 g DW (mean ± standard deviation, $n = 3$). Percent contribution to total phenolic content is in parentheses. Values with no letters in common are significantly different ($p < 0.05$).

	Free	Bound	Total
KanQueen	254.7 ± 10.0g (30.3)	586.2 ± 53.9cd (69.7)	841 ± 62.3b
Caledonia	307.0 ± 21.0fg (35.9)	548.8 ± 1.8d (64.1)	856 ± 19.5b
Foster	332.5 ± 24.8fg (35.7)	598.5 ± 24.6cd (64.3)	931 ± 40.7b
Cham1	398.6 ± 8.5ef (37.6)	662.4 ± 35.4c (62.4)	1061 ± 36.2a
Roane	499.5 ± 27.0de (46.2)	581.9 ± 49.0cd (53.8)	1081 ± 52.2a
Stoa	459.1 ± 18.5de (41.8)	639.7 ± 34.9cd (58.2)	1099 ± 19.1a

Table 3
Phenolic acid composition of six diverse varieties of wheat. Values expressed as μmol phenolic acid/100 g DW (mean ± standard deviation, $n = 3$). Percent contribution to total phenolic acid content is in parentheses. Values with no letters in common within each column are significantly different ($p < 0.05$); nd – not detected.

	Free	Soluble-conjugated	Insoluble-bound	Total
Ferulic acid				
Caledonia	8.7 ± 0.7b (2.8)	30.5 ± 2.7a (9.8)	271.5 ± 5.1b (87.4)	310.8 ± 7.2b
Cham1	11.6 ± 0.7b (3.3)	11.8 ± 1.1b (3.4)	326.7 ± 29.8b (93.3)	350.1 ± 28.9b
Foster	4.3 ± 1.4c (1.3)	12.3 ± 2.1b (3.8)	307.7 ± 14.2b (94.9)	324.2 ± 15.6b
KanQueen	4.4 ± 0.6c (0.9)	9.6 ± 1.0b (1.9)	482.1 ± 90.3a (97.2)	496.1 ± 90.2a
Roane	20.1 ± 0.5a (6.2)	11.4 ± 0.9b (3.6)	290.4 ± 36.3b (90.2)	321.8 ± 36.4b
Stoa	10.2 ± 1.3b (2.7)	11.5 ± 0.7b (3.0)	361.8 ± 19.6b (94.3)	383.5 ± 21.2b
p-Coumaric acid				
Caledonia	25.4 ± 4.3a (48.6)	10.0 ± 0.6a (19.1)	16.9 ± 0.5b (32.3)	52.3 ± 4.0a
Cham1	10.2 ± 0.6b (30.4)	7.5 ± 0.2bc (22.3)	15.9 ± 1.3b (47.3)	33.5 ± 0.5c
Foster	9.2 ± 1.8b (26.0)	9.2 ± 1.3ab (26.0)	17.0 ± 1.5b (48.0)	35.5 ± 4.4c
KanQueen	9.5 ± 1.2b (20.4)	8.0 ± 0.4b (17.2)	29.0 ± 2.4a (62.4)	46.5 ± 1.5ab
Roane	14.4 ± 1.1b (35.0)	7.7 ± 0.2b (18.7)	19.0 ± 1.5b (46.3)	41.1 ± 2.3bc
Stoa	11.2 ± 1.7b (32.4)	7.4 ± 0.1c (21.4)	16.0 ± 1.6b (46.2)	34.7 ± 1.2c
Syringic acid				
Caledonia	nd	11.5 ± 0.3a (78.8)	3.1 ± 0.6b (21.2)	14.6 ± 0.3a
Cham1	nd	4.9 ± 0.8c (54.4)	4.1 ± 0.7b (45.6)	9.0 ± 1.5b
Foster	nd	8.7 ± 2.5ab (100)	nd	8.7 ± 2.5b
KanQueen	nd	5.7 ± 1.5bc (36.8)	9.8 ± 2.3a (63.2)	15.5 ± 3.3a
Roane	nd	6.3 ± 0.7bc (60.0)	4.2 ± 0.6b (40.0)	10.5 ± 1.3ab
Stoa	nd	7.6 ± 0.1bc (65.0)	4.1 ± 0.6b (35.0)	11.7 ± 0.6ab
Vanillic acid				
Caledonia	nd	8.3 ± 2.0a (100)	nd	8.3 ± 2.0a
Cham1	nd	6.5 ± 0.5ab (100)	nd	6.5 ± 0.5ab
Foster	nd	7.8 ± 1.6a (100)	nd	7.8 ± 1.6a
KanQueen	nd	4.3 ± 1.4b (100)	nd	4.3 ± 1.4b
Roane	nd	7.6 ± 0.5a (100)	nd	7.6 ± 0.5a
Stoa	nd	6.0 ± 0.2ab (100)	nd	6.0 ± 0.2ab
Caffeic acid				
Caledonia	nd	nd	4.4 ± 1.1c (100)	4.4 ± 1.1c
Cham1	nd	nd	7.4 ± 0.9bc (100)	7.4 ± 0.9bc
Foster	nd	nd	5.9 ± 0.5bc (100)	5.9 ± 0.5bc
KanQueen	nd	nd	11.6 ± 0.4a (100)	11.6 ± 0.4a
Roane	nd	nd	7.5 ± 1.0b (100)	7.5 ± 1.0b
Stoa	nd	nd	8.2 ± 2.0b (100)	8.2 ± 2.0b

3.3. Carotenoid content

Total carotenoid content ranged from 148 (KanQueen) to 271 (Foster) μg/100 g DW. Lutein content was the highest amongst the carotenoids tested and ranged from 67.4 (KanQueen) to 211.1 (Foster) μg/100 g DW. Zeaxanthin content ranged from 25.3 (Cham1) to 52.7 (Caledonia) μg/100 g DW. β-Cryptoxanthin content ranged from 11.8 (Caledonia) to 19.5 (Roane) μg/100 g DW. None of the β-cryptoxanthin contents were significantly different ($p > 0.05$). β-Carotene content ranged from 17.6 (Cham1) to 36.2 (Foster) μg/100 g DW (Table 4). α-Carotene was not detected in any variety tested.

3.4. Vitamin E content

Total vitamin E content ranged from 13.4 (Cham1) to 19.6 (KanQueen) μg/g DW (Table 5). In all samples, β-tocotrienol was the predominant form of vitamin E. β-Tocotrienol content ranged from 8.6 (Roane) to 11.9 (Caledonia) μg/g DW. Mainly α- and β-tocopherols and -tocotrienols were detected in the samples. Four of the six varieties of wheat contained γ-tocopherol. The content of γ-tocopherol was low and ranged from 0.1 (Cham1) to 0.9 (KanQueen) μg/g DW. α-Tocopherol content ranged from 0.7 (Cham1) to 5.2 (KanQueen) μg/g DW.

Table 4
Carotenoid (lutein, zeaxanthin, β-cryptoxanthin, and β-carotene) content and distribution of wheat varieties (mean ± standard deviation, $n = 3$). Values with no letters in common within each column are significantly different ($p < 0.05$).

	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene
KanQueen	67.4 ± 21.5c	27.8 ± 1.4c	15.9 ± 2.2	19.7 ± 3.6b
Stoa	97.6 ± 5.4c	35.1 ± 1.7b	16.0 ± 2.5	17.9 ± 4.4b
Roane	119.0 ± 32.8bc	44.1 ± 6.6ab	19.5 ± 3.4	23.6 ± 1.2b
Caledonia	173.5 ± 22.4ab	52.7 ± 3.8a	11.8 ± 2.2	23.1 ± 7.5b
Cham1	207.6 ± 33.6a	25.3 ± 2.6c	17.4 ± 3.2	17.6 ± 3.6b
Foster	211.1 ± 5.8a	39.8 ± 0.1b	14.5 ± 3.3	36.2 ± 8.2a

3.5. Oxygen radical absorbance capacity (ORAC)

The free ORAC ranged from 1958 (KanQueen) to 3749 (Stoa) μmol Trolox equivalents/100 g DW. The bound ORAC ranged from 3190 (KanQueen) to 5945 (Roane) μmol Trolox equivalents/100 g DW. The total ORAC of the wheat samples ranged from 5148 (KanQueen) to 9616 (Stoa) μmol Trolox equivalents/100 g DW (Table 6). Total phenolic content was correlated with ORAC ($R^2 = 0.810$; $p < 0.001$).

4. Discussions

4.1. Total phenolic content

Phenolics are compounds with one or more aromatic ring and one or more hydroxyl groups (Liu, 2003). Phenolic compounds from the insoluble-bound fraction of whole wheat are of particular importance because of their ability to scavenge free radicals and prevent the oxidation of biologically important molecules in the colon (Adom & Liu, 2002; Liyana-Pathirana & Shahidi, 2006). The total phenolic contents we reported here are similar to the total

Table 5
Vitamin E content of wheat varieties. Values expressed as μg/g DW (mean ± standard deviation, $n = 3$). Values with no letters in common within each column are significantly different ($p < 0.05$); nd – not detected.

	Tocopherols				Tocotrienols				Total VE
	α	β	γ	δ	α	β	γ	δ	
Caledonia	0.7 + 0.1c	1.8 + 0.2c	0.2 + 0.03c	nd	0.4 + 0.1b	7.2 + 0.9b	nd	nd	10.2 + 1.3b
Roane	1.2 + 0.3bc	2.9 + 0.6ab	0.2 + 0.01b	nd	0.5 + 0.1b	8.6 + 1.2ab	nd	nd	13.4 + 2.1ab
Cham1	0.7 + 0.1c	1.2 + 0.3c	0.1 + 0.03c	nd	1.3 + 0.1a	9.2 + 1.0ab	nd	nd	13.8 + 2.1ab
Stoa	1.9 + 0.3bc	2.1 + 0.3bc	nd	nd	0.6 + 0.1b	9.9 + 0.9ab	nd	nd	14.5 + 1.7ab
Foster	4.2 + 2.0ab	1.3 + 0.1c	1.3 + 0.1a	nd	1.4 + 0.2a	10.0 + 0.9ab	nd	nd	18.1 + 2.6a
KanQueen	5.2 + 2.0ab	1.5 + 0.4c	0.9 + 0.1b	nd	1.0 + 0.2a	11.2 + 1.3a	nd	nd	19.8 + 0.8a

Table 6
ORAC values of wheat varieties (mean ± standard deviation, $n = 3$). Percent contribution to total ORAC is in parentheses. Values with no letters in common are significantly different ($p < 0.05$).

	Free ORAC	Bound ORAC	Total ORAC[A]
KanQueen	1958 ± 251g (38.0)	3190 ± 687fg (62.0)	5148 ± 486def
Caledonia	2703 ± 653fg (39.4)	4159 ± 316f (60.6)	6862 ± 342cd
Cham1	2552 ± 394g (35.6)	4594 ± 577ef (64.4)	7174 ± 962bc
Foster	3545 ± 426fg (47.2)	3960 ± 631f (52.8)	7505 ± 424bc
Roane	2694 ± 192fg (31.2)	5945 ± 754de (68.8)	8639 ± 830ab
Stoa	3749 ± 423fg (39.0)	5867 ± 352de (61.0)	9616 ± 752a

[A] Sum of free and bound ORAC.

phenolic content values for 11 diverse varieties of whole wheat reported earlier (Adom et al., 2003), and ranged from 710 to 860 μmol gallic acid equivalents/100 g FW. Moore et al. (2005) reported the total phenolic content of eight Maryland-grown soft wheat samples, ranged from 235 to 470 μmol gallic acid equivalents/100 g. These values were significantly lower than those reported in the present study and Adom et al. (2003). This may be due to differences in extraction procedures. Because no alkali hydrolysis and further extraction was performed, the authors only extracted phenolic compounds from the free fraction. This would lead to an underestimation of the total phenolic content. The total phenolic content values reported by Moore et al. (2005) are similar to the free phenolic content values reported in this study.

The total phenolic content of wheat has also been reported in ferulic acid equivalents. Mpofu et al. (2006) reported that the average total phenolic content of six wheat genotypes was 958 μmol ferulic acid equivalents/100 g. Liyana-Pathirana and Shahidi (2006) reported that the total phenolic content of soft and hard wheat was 1966 and 1780, respectively. The bound fraction contributed 56% and 62% to the total phenolic content, respectively. This finding is consistent with the percent contribution from the bound fraction in this study. On average, the bound fraction accounted for 62% of the total phenolic content.

4.2. Phenolic acid composition

Phenolic acids are derivatives of hydroxybenzoic acid or hydroxycinnamic acid. Ferulic acid, a hydroxycinnamic acid derivative, is the predominant phenolic acid found in whole grains (Mattila, Pihlava, & Hellstrom, 2005; Sosulski, Krygier, & Hogge, 1982) and whole wheat (Moore et al., 2005; Siebenhandl et al., 2007). Sosulski et al. (1982) reported that the total ferulic acid content was 32.7 μmol ferulic acid/100 g with the bound ferulic acid content accounting for 92% of the total ferulic acid content. Adom et al. (2003) reported that the total ferulic acid content of 11 diverse varieties of whole wheat ranged from 147.7 to 303.0 μmol ferulic acid/100 g with the bound ferulic acid accounting for more than 97% of the total ferulic acid content. Moore et al. reported that the total ferulic acid content of eight Maryland-grown soft wheat samples ranged from 234.8 to 320.0 μmol ferulic acid/100 g with the bound ferulic acid accounting for more than 89% of the total ferulic acid content (Moore et al., 2005). Li, Shewry, and Ward (2008) reported that the total ferulic acid content of spring wheat and winter wheat were 209.2 and 205.4 μmol ferulic acid/100 g, respectively.

Mattila et al. (2005) reported that the total ferulic acid content of whole-wheat flour was 458.3 μmol ferulic acid/100 g DW. Mpofu et al. (2006) reported that the total ferulic acid content of hard spring wheat ranged from 191.0 to 227.1 μmol ferulic acid/100 g. Siebenhandl et al. (2007) reported that the total ferulic acid content of a purple pericarp wheat was 438.5 μmol ferulic acid/100 g FW. The contribution from the bound fraction to the total ferulic acid content was not reported in any of these studies.

In this study, the total ferulic acid content ranged from 310.8 to 496.1 μmol ferulic acid/100 g DW. The bound fraction contributed 87–97% to the total ferulic acid content. These findings are similar to those previously reported (Adom et al., 2003; Mattila et al., 2005; Moore et al., 2005; Mpofu et al., 2006; Siebenhandl et al., 2007).

Other phenolic acids were also found in significant quantities in whole wheat. The hydroxycinnamic acid derivatives are more prevalent than the hydroxybenzoic acid derivatives. The second most abundant phenolic acid found in whole wheat is *p*-coumaric acid. Moore et al. (2005) reported the *p*-coumaric acid content of eight varieties of Maryland-grown soft wheat. The average total *p*-coumaric acid content was 7.4 μmol/100 g. The bound fraction accounted for nearly 90% of the total *p*-coumaric acid content. Mattila et al. (2005) reported the total *p*-coumaric acid and total caffeic contents of whole wheat. The total *p*-coumaric acid and total caffeic acid contents of whole wheat were 22.5 and 20.5 μmol/100 g DW, respectively. Mpofu et al. (2006) reported the total *p*-coumaric acid and total caffeic acid content of six genotypes of wheat. The average total *p*-coumaric acid and caffeic acid contents were 18.9 and 5.6 μmol/100 g, respectively. Siebenhandl et al. (2007) reported that the total *p*-coumaric acid content of purple pericarp wheat was 14.8 μmol/100 g DW. Li et al. (2008) reported that the total *p*-coumaric acid content of spring wheat and winter wheat was 6.7 and 9.6 μmol *p*-coumaric acid/100 g, respectively. However, the contribution of *p*-coumaric acid from the bound fraction varied between the two types of wheat. The bound fraction of winter wheat accounted for 63% of the total *p*-coumaric acid content, whilst spring wheat accounted for 37%. Winter wheat contained 0.2 μmol caffeic acid/100 g though no caffeic acid was found in the spring wheat (Li et al., 2008). All of the caffeic acid in the winter wheat was from the free fraction.

The *p*-coumaric acid contents reported in the present study are higher than those previously reported in literature. The percent contribution to the total *p*-coumaric acid content was most similar to that reported by Li et al. (2008). In this study, the bound *p*-coumaric acid content from the winter wheat varieties contributed 47.3% to the total *p*-coumaric acid content compared to 63.3%, as reported by Li et al. (2008). Caffeic acid contents reported across

a number of studies appear to be more variable than ferulic acid and *p*-coumaric acid contents. In this study, the average caffeic acid content was 7.5 μmol/100 g DW, with the bound fraction contributing all of the caffeic acid found. This value agrees most with that reported by Mpofu et al. (2006).

Hydroxybenzoic acid derivatives have also been reported in whole wheat. Moore et al. (2005) reported that the vanillic and syringic acid contents of eight varieties of Maryland-grown soft wheat were 6.2 and 5.3 μmol/100 g. The soluble-conjugated fraction contributed the most to the total contents of both vanillic acid and syringic acid, 51% and 65.6%, respectively. Only two of the eight varieties had any syringic acid in the free fraction. The bound fraction contributed 38.5% and 34.3% to the total contents of vanillic acid and syringic acid, respectively. No *p*-hydroxybenzoic acid was detected in this study. Mattila et al. (2005) reported the content of hydroxybenzoic acid derivatives from whole wheat. The total vanillic, syringic, and *p*-hydroxybenzoic acid contents were 8.9, 6.6, and 5.4 μmol/100 g DW, respectively (Mattila et al., 2005). Mpofu et al. (2006) reported that the total content of vanillic and syringic acids, were 5.4 and 5.7 μmol/100 g, respectively. Siebenhandl et al. (2007) reported that the total vanillic acid content of purple pericarp wheat was 20.9 μmol/100 g. Li et al. (2008) reported that the total vanillic and syringic acid contents of winter wheat were 12.4 and 8.9 μmol/100 g. The soluble-conjugated fraction contributed the most to the total vanillic and syringic acid contents of winter, 67.5% and 61.9%, respectively. Li et al. (2008) also reported that the total vanillic and syringic acid contents of spring wheat were 11.6 and 8.8 μmol/100 g, respectively. The soluble-conjugated fraction contributed the most to the total vanillic and syringic acid contents of spring wheat, 71.8% and 63.6%, respectively.

The total content of hydroxybenzoic acid derivatives reported in the present study is consistent with previously reported values. The average total vanillic acid and syringic acid contents were 6.8 and 11.7 μmol/100 g DW. However, there were slight differences in percent contribution from the various fractions. In the present study, no vanillic acid was detected in the free or bound fractions. Further, no syringic acid was detected in the free fraction, which is consistent with the results reported by Moore et al. (2005). No *p*-hydroxybenzoic acid was detected in any variety of wheat, which is also consistent with data reported by Moore et al. (2005), but not consistent with that reported by Mattila et al. (2005).

4.3. Carotenoid content

Hentschel et al. (2002) reported that the carotenoid content of four durum wheat samples averaged to roughly 200 μg/100 g. In this study, no β-carotene was detected. Further, the total carotenoid content of the durum wheat sample was 240 μg/100 g. Adom et al. (2003) reported the carotenoid content of 11 diverse varieties of whole wheat. However, the β-carotene content of each sample was not reported. The lutein, zeaxanthin, and β-cryptoxanthin values reported in this study are similar to those reported by Adom et al. (2003), though the values are slightly higher and the range of values is narrower. Moore et al. (2005) reported the carotenoid content of eight Maryland-grown soft wheat samples. The carotenoid values reported in that study are similar to those reported in this study. However, β-cryptoxanthin content was not reported. Here the carotenoid content of four different carotenoids is reported. Hidalgo, Brandolini, Pompei, and Piscozzi (2006) reported the carotenoid content of 54 cultivars of Einkorn wheat, six durum wheat cultivars, and five bread wheat cultivars grown across Europe. The average total carotenoid content of the durum wheat, bread wheat, and Einkorn wheat varieties was 320, 195, and 841 μg/100 g DW, respectively.

The total carotenoid content of the six diverse varieties of wheat reported in this study ranged from 148 to 271 μg/100 g DW. However, one variety of durum wheat (Cham1) was used in the present study. The values reported for lutein and β-carotene in this study are also in agreement with those reported by Hidalgo et al. (2006) and Adom et al. (2005).

4.4. Vitamin E content

All varieties of wheat contained more tocotrienols than tocopherols (Table 3). The tocotrienol:tocopherol ratio ranged from 1.9 (KanQueen) to 5.3 (Cham1). Panfili et al. (2003) reported that the average tocotrienol:tocopherol ratio of soft wheat and durum wheat was 1.9 and 3.3, respectively. Here, we report that the average tocotrienol:tocopherol ratio of soft wheat and durum wheat was 3.0 and 5.3, respectively.

Panfili et al. (2003) reported the vitamin E content of durum and soft wheat. Total vitamin E content for durum wheat and soft wheat was 60.6 and 74.3 μg/g DW, respectively. In both cases, β-tocotrienol was the predominant form of vitamin E. As Panfili et al. (2003) noted, there are no standardised methods for the extraction and analysis of vitamin E from cereals. Panfili et al. (2003) showed that a methanol extraction or a solvent extraction without prior saponification resulted in lower recovery of vitamin E when compared to the saponification followed by organic solvent extraction. Moore et al. (2005) determined the tocopherol content of eight Maryland-grown soft wheat samples using ESI-MSMS. Tocotrienol content was not reported. Only α-tocopherol was detected in these wheat samples. Not being able to detect β-tocopherol may be due to the extraction procedure, in which, methanol/THF 1:1 v/v was used with no saponification. Hidalgo et al. (2006) reported the vitamin E content of 54 cultivars of Einkorn wheat, six durum wheat cultivars, and five bread wheat cultivars grown across Europe. The total vitamin E content for the durum wheat, bread wheat, and Einkorn wheat varieties was 50.5, 61.5, and 78.0 μg/g DW, respectively. These values are at least 2-fold higher than those reported in this study.

The vitamin E contents reported in the present study are lower than previously reported values (Hidalgo et al., 2006; Lampi, Nurmi, Ollilainen, & Piironen, 2008; Panfili et al., 2003). However, the distribution of tocopherols and tocotrienols reported in the present study is consistent with previously reported values. β-Tocotrienol was the predominant form of vitamin E accounting for roughly half of the total vitamin E content. γ-Tocopherol was detected in five of the six varieties analysed. Grela (1996) reported the presence of γ-tocopherol in durum wheat. The present study is consistent with the data reported by Grela, finding γ-tocopherol not only in durum wheat, but also in hard and soft red wheat. The ability to detect γ-tocopherol in wheat may be due to the efficiencies of the extraction and analytical methods.

4.5. Oxygen radical absorbance capacity

The antioxidant activity of whole wheat extracts have been determined using previously reported antioxidant activity assays (Adom et al., 2003, 2005; Moore et al., 2005; Pellegrini et al., 2006; Siebenhandl et al., 2007). Comparing the antioxidant capacity of the wheat varieties used in these studies is difficult because the different antioxidant capacity assay have not been standardised (Fardet, Rock, & Remesy, 2008).

Moore et al. (2005) reported the ORAC of eight Maryland-grown soft wheat samples. ORAC values ranged from 3290 to 4770 μmol Trolox equivalents/100 g. These values were similar to the free ORAC values reported in this study and much lower than the total ORAC values reported. Total phenolic content was correlated with the ORAC ($r = 0.908$; $p = 0.01$). However, Moore et al. (2005) did not

report the ORAC contribution from the free and bound fraction. Liyana-Pathirana and Shahidi (2006) reported that the bound fraction from hard and soft winter wheat contributed 87.4% and 86.6% to the total ORAC, respectively.

In the present study, the total ORAC values of six diverse varieties of wheat ranged from 5148 to 9616 μmol Trolox equivalents/100 g DW. The free ORAC ranged from 1958 to 3749 μmol Trolox equivalents/100 g DW. The bound ORAC ranged from 3190 to 5945 μmol Trolox equivalents/100 g DW. Three of the six varieties of wheat had bound ORAC values that were significantly different ($p < 0.05$) from the free ORAC (Table 5). The bound fraction contributed 52.8–68.8% of the total ORAC.

4.6. Summary

Whole grain consumption is associated with reduced risk of chronic diseases including cardiovascular disease, type II diabetes, obesity, and cancer. Here the phytochemical content and antioxidant activity of six diverse varieties of whole wheat are reported. The bound fraction contributed 53.8–69.7% of the total phenolic content of the wheat varieties analysed. Ferulic acid was the predominant phenolic acid found in whole wheat. Other phenolic acids, *p*-coumaric acid, syringic acid, vanillic acid, and caffeic acid were also detected. The carotenoid content is also reported in this study. Lutein was the predominant carotenoid found in the whole wheat varieties analysed. Zeaxanthin, β-carotene, and β-cryptoxanthin were also detected. The vitamin E content of whole wheat is reported in the present study. Mainly α- and β-tocopherols and -tocotrienols were found in all varieties of whole wheat though γ-tocopherol was detected in all but two varieties. β-Tocotrienol was the predominant form of vitamin E found in all varieties of whole wheat. The antioxidant activity was assessed using the ORAC assay. The ORAC of the free fraction ranged from 1958 to 3749 μmol Trolox equivalents/100 g DW. The ORAC of the bound fraction ranged from 3190 to 5945 μmol Trolox equivalents/100 g DW. Total phenolic content was correlated with oxygen radical absorbance capacity ($R^2 = 0.810$; $p < 0.001$). The phytochemicals found in whole grains may be responsible for the health benefit of whole grain consumption.

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